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We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. We have developed a novel strategy to establish the protozoan parasite *T. gondii* as the next generation vector for breast cancer gene therapy. The significant innovative aspect of this approach is the promise of this strategy to deliver a novel vector for breast cancer gene therapy that is potentially superior to the current vectors under current development and refinement. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to safely target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models. In this reporting period we have found that the cytosine deaminase (CD) and thymidine kinase (TK) markers expressed in *T. gondii* produce a bystander killing effect on both human fibroblasts and SKBR3 tumor cells *in vitro*. We also report construction of a highly attenuated uracil auxotrophic *T. gondii* parasite strain for safely targeting gene therapy to breast cancer. Using trifunctional ezyme expression plasmids we have co-expressed both the TK and CD markers in transgenic *T. gondii*. We are examining the expression of HSV TK and bacterial CD in attenuated *T. gondii*, response to ganciclovir and 5-fluorocytosine, as well as methods for targeting this vector to breast cancer cells overexpressing HER2/neu. High affinity antibodies to examine a bispecific targeting approach have been obtained.

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Introduction

We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. We have developed a novel strategy to establish the protozoan parasite *T. gondii* as the next generation vector for breast cancer gene therapy. The significant innovative aspect of this approach is the promise of this strategy to deliver a novel vector for breast cancer gene therapy that is superior to the current vectors under current development and refinement. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models.

Body

The statement of work is reiterated below for reporting period:

Task 3: Months 11-16: Opsonize parasites and examine targeting of parasite via bispecific antibody. Examine killing of breast cancer cells using 5-FC and ganciclovir.

Task 4: Months 16-20: Develop, clone and document single chain antibody variable region fragment (sFv) that recognizes HER2/neu

Research Accomplishments associated with each task:

Task 3: Months 11-16: Opsonize parasites and examine targeting of parasite via bispecific antibody. Examine killing of breast cancer cells using 5-FC and ganciclovir.

We have now examined the bystander effect for transgenic T.~gondii expressing thymidine kinase (TK) or bacterial cytosine deaminase in SKBR3 cells that overexpress HER2/neu. Assessment by the DEAD red cell assay and by Trypan blue exclusion assays showed that infection of approximately 10 to 20% of the cell population with TK or CD expressing T.~gondii, followed by treatment of the cell culture with 5 μ M ganciclovir, or 50 μ M 5-fluorocytosine, respectively, resulted in greater than 90% killing of the uninfected cell population after a 24 h incubation.

We have obtained transgenic *T. gondii* that stably express both enzyme activities in *T. gondii*. These parasites are sensitive to both prodrugs (ganciclovir and 5-fluorocytosine). As expected, we find that the bystander effect is greater in the parasites which express both suicide enzymes. Only 5 to 10% of the SKBR3 cells need to be infected in order to kill >90% of the cells after treatment with ganciclovir and 5-fluorocytosine in the *in vitro* assays.

Related to the task of constructing transgenic *T. gondii* expressing both TK and CD is the development of a "safe" or "safer" strain of *T. gondii* which could be more appropriately used and controlled in the *in vitro* and *in vivo* targeting studies. We report that we have created a uracil auxotroph mutant of *T. gondii* that has properties which make it an ideal vector for targeting cancer cells. This mutant is completely attenuated in both immune competent and immunocompromised

mice. The mutant invades host cells normally and will express proteins for several days; however, this mutant does not replicate *in vitro* or *in vivo* in the absence of uracil supplementation. For these reasons we plan to extend tasks 3 and 4 to include the development of the expression of TK and CD markers, at useful levels, in the uracil auxotroph mutant strain of *T. gondii*. We have initiated experiments to express the TK and CD marker in this attenuated mutant but do not yet have significant data to report.

We have made significant progress on task 3,however, task 3 is not yet fully completed. As reported we have produced approximately 20 mg of anti-HER2/neu Mab 520C9 and the resulting Fab' which is required for the construction of the bispecific targeting antibody. However, the antibody (Mab6A8) we previously obtained to the major surface antigen of *T. gondii*, the P30 antigen (Kasper and Ware, 1987), did not have sufficient avidity/affinity to warrant its use in a bispecific construct. We have now successfully obtained high affinity/avidity anti-P30 polyclonal serum that can produce an improved bispecific antibody. Sufficient IgG fraction has been obtained and we anticipate that the construction of the bispecific will be completed.

Task 4: Months 16-20: Develop, clone and document single chain antibody variable region fragment (sFv) that recognizes HER2/neu

This task will initiate after we complete task 3.

Problems in accomplishing tasks:

We have made significant progress in accomplishing the tasks and we have also experienced some difficulties in this work. Some of the scientific based difficulties were mentioned above. In the past we have had some personnel problems, which were reported in the last period. This past period we had the circumstance that a technician became pregnant and according to both laboratory and Dartmouth policy could not work with Toxoplasma for several months. This has somewhat slowed our progress. On the other hand, the excellent personnel news is that I have filled the project with two new personnel, Ph.D. students in our Molecular and Cell Biology Program, students who have recently joined my lab. One of the students will work on the project full time this year and next, while the other will work on the project with more than 50% of effort. These additions will greatly accelerate our progress in this project.

Key Research Accomplishments to date

- Co-expression of cytosine deaminase and thymidine kinase in T. gondii
- TK transgenic T. gondii exhibit the bystander effect with ganciclovir treatment
- CD transgenic *T. gondii* exhibit the bystander effect with 5-fluorocytosine treatment
- CD and TK transgenic *T. gondii* exhibit the bystander effect on HER2/neu overexpressing SKBR3 tumor cells
- High avidity/affinity IgG antibody to the major surface protein of *T. gondii* was obtained and purified
- A uracil auxotroph mutant strain of *T. gondii* was developed and found to be completely avirulent in both immune competent as well as in severely immune devifient mice

Reportable Outcomes

Manuscript in preparation B.A. Fox & D.J. Bzik. A potentially safe *Toxoplasma gondii* vector based on uracil auxotrophy.

Conclusions

Both the thymidine kinase and cytosine deaminase genetic markers have been expressed in transgenic T. gondii and express the "bystander" effect in both human fibroblast and HER2/neu overexpressing SKBR3 cell lines. That is, that neighboring cells to those expressing CD or TK can be killed by the toxic products formed following treatment with 5-fluorocytosine or ganciclovir, respectively. Transgenic T. gondii have been obtained that co-express the TK and CD transgenes. The development of a targeting strategy employing bispecific antibody or stable genetic equivalents is being developed to target the TK and CD activities to breast cancer tissue(s) in vitro and in vivo. A T. gondii uracil auxotroph was developed and found to be avirulent in immune competent and immune deficient mice. The parasite vector may be an ideal vector, or ideal prototype for our targeting strategies. This avirulent vector should provide a "safer" vector for use in the targeting studies, as well as a parasite that may be more useful in a variety of ways. This attenuated mutant is important when considering the in vivo studies. We can now perform studies in immunocompromised mice, or normal mice, and we can have significant latitude on timing of drug delivery or targeting effects. In addition, studies at direct inoculation of mice tumors is now possible using this avirulent mutant.

References

1. Ware, P.L. and L. H. Kasper. (1987) Strain specific antigens of *Toxoplasma gondii*. *Inf Imm.* 55:778-783.

Appendices